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Gene Expression Is Required for Correct Axon Guidance

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Summary

As axons form connections in the developing brain, they often change course before they reach their final target. An outstanding question concerning changes in axon guidance is whether autonomous gene expression directs axons to their targets. If autonomous gene expression is required during axon guidance, then at least some aspects of guidance may be irreversible. Once commissural axons cross the midline in the central nervous system (CNS), they usually make guidance decisions that differ from those made on the ipsilateral side. Here I explore whether a change in gene expression is involved in postcommissural axon guidance. Grasshopper serotonergic neurons were microinjected with a fluorescent tracer dye and either a translation blocker or a transcription blocker. After 24 hr of development, the guidance decisions of these microinjected axons were assayed. If the growth cones had already made a postcommissural guidance choice after crossing the midline, they continued growth even when gene expression was inhibited. If growth cones had just crossed the midline and not yet reached the choice point, they made a distinct guidance error when gene expression was inhibited. These results show that there is a discrete step in which gene expression plays a critical role in postcommissural axon guidance.

Results and Discussion

The function of the central nervous system (CNS) depends upon the correct connectivity between neurons. Much of this connectivity is established early in development and is specified by the directed growth of axons. The tip of a growing axon, or growth cone, is thought to contain receptors that bias migration through a complex field of embryonic morphogenetic signals [1]. A number of experiments utilizing isolated growth cones have indicated that all of the necessary guidance machinery is localized to the growth cone [2, 3]. In vitro evidence does exist for local protein synthesis in growth cones [4–6]. In vivo bath application of transcription blockers has also indicated that gene expression is required for very specific axon guidance events [7]. Experiments in the CNS are likely to yield much more complex results than those in vitro because growth cones are likely to be presented with a multitude of paths at any point.

Two serotonergic neurons, s1 and s2, are born in each hemiganglion of the grasshopper ventral nerve cord [3, 8]. Both of these neurons send axons across the midline, after which they pause on the contralateral connective [3]. The axons then extend anteriorly, but s2 stays on the intersegmental connective and s1 branches in the lateral synaptic neuropil. Like most commissural axons, s1 and s2 ignore targets on the ipsilateral side that are recognized on the contralateral side. The current hypothesis to explain this behavior is that a fundamental guidance change occurs in commissural neurons once they cross the midline [9]. The function of members of the *roundabout* gene family have been linked to this change [1]. Although it is clear that axons do change their guidance properties upon crossing the midline [10] and that signals from the midline and/or intrinsic timing are likely to be involved [3, 11–13], it is unknown whether gene expression is involved in this change. Here I test whether microinjected transcription or translation blockers alter axon guidance in situ. These experiments show that axon guidance requires autonomous gene expression.

To assay normal growth cone migration in the grasshopper, I established a microinjection/culture preparation. The thoracic s1/s2 serotonergic neurons in isolated ventral nerve cords from staged embryos were microinjected with fluorescent dextran. The position of the growth cone of the microinjected cell was photodocumented (Figure 1A) and then allowed to develop in situ for 24 hr. The preparations were then fixed and in many cases stained for serotonin uptake that begins when the growth cones arrive at the contralateral connective [3]. The progress of the growth cone of the injected neuron can be scored (Figures 1B–1D). At the time of initial microinjection in Figure 1A, the growth cone was on the ipsilateral connective and heading toward the midline. After 24 hr, the growth cone had reached the contralateral connective, turned, and was heading into the neuropil. The contralateral s1 serotonergic growth cone (arrow in Figure 1C) had migrated slightly farther than the injected side (arrow in Figure 1D). A slight delay in migration was seen in many of the injected cells as compared to the control side. In Figure 1E, the growth cone was at a slightly earlier stage than that in Figure 1A and had, after 24 hr in culture, migrated to the contralateral connective and just begun serotonin uptake activity. Figure 1F shows the development in situ of a growth cone that had already turned on the connective when injected. After the 24 hr culture period, it had entered the synaptic neuropil and begun to branch. For these and all experiments described here, the position of the growth cone at injection is taken as the most forward part of a contiguous axon. The position of each growth cone was then classified as on or between three landmarks: the ipsilateral connective, the midline, and the contralateral connective.

Microinjection with the transcription blocker actinomycin D blocked axon migration across the midline (Figures 2A and 2B). This is also true for translation blockers and may be true for all young neurons (see below). Growth cones either stalled for the 24 hr culture period

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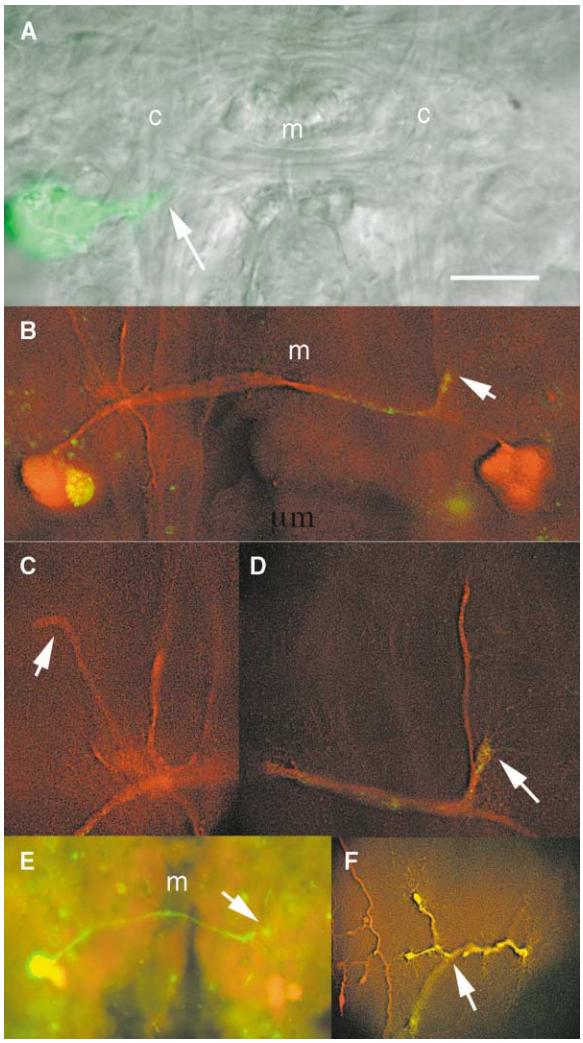

Figure 1. Growth Cone Progression of Dye-Microinjected Serotonergic Neurons

Grasshopper serotonergic neurons were microinjected with FITC-conjugated lysinated dextran (10k), photo documented to record the growth cone position, and allowed to develop for 24 hr. Preparations were then fixed, stained for serotonin uptake, and photographed. (A) A live microinjected serotonergic growth cone (arrow) is just entering the posterior commissure and is growing toward the midline (m). (B) After 24 hr, the same growth cone as above was fixed and stained for serotonin uptake (red). The growth cone (arrow) has crossed the midline and turned toward the contralateral target neuropil. (C) A higher magnification photograph of the ipsilateral s1 noninjected growth cone, which has begun to bifurcate while the microinjected side (D) is slightly delayed. In most of these experiments, the microinjected side as compared to the control side is delayed an estimated 3–4 hr. (E) A microinjected serotonergic neuron has reached the point at which serotonin uptake begins. (F) A growth cone at about the same stage as that in (D) at microinjection has bifurcated and begun to form secondary branches after 24 hr. The scale bar in (A) represents 10 μm for (A), (B), and (F), 5 μm for (C) and (D), and 20 μm for (E).

(Figures 2A and 2B) or slightly retracted (my unpublished data). However, if the growth cones had crossed the midine but not yet reached the turning point at the time of injection, all continued to migrate but made an incorrect choice of direction (Figures 2C–2E). Instead of migrating anteriorly, growth cones extended either posteriorly on the connective or in a posterior/lateral manner (see translation-blocker experiment below). If the growth cone had already reached the connective or made the turn (see Figure 2G) at the time of injection, growth continued in the correct direction (Figures 2F and 2H). These data indicate that once growth cones have crossed the midline, blocking transcription does not affect general axon progression. Presumably, the mRNA/protein present at the time of microinjection is sufficient for the 24 hr culture period. Incorrect axonal migration was only seen in cases when the axon had not yet reached the contralateral connective prior to injection. This indicates that transcription is required for correct axon path choice and that this transcription occurs before the choice point is reached. Once axons made the choice, they continued to grow in the correct direction (Figures 2F and 2H). However, in all of these latter cases, no s1-type bifurcations in the neuropil were seen, as they were in Figure 1F. In controls, more s1's were injected than s2's, presumably because the cell body was accessible. Where serotonin staining was done, the axon of the microinjected cell (Figure 2F, arrow) grows in the connective instead of into the neuropil (compare left and right sides of Figure 2F). This can be interpreted as a block of the s2 branching pattern. Thus, although blocking transcription does not block anterior migration of serotonergic growth cones, it may affect specific target choice. The affected s1 may have either been blocked from differentiating into the neuropil-branching pattern or acquired the abdominal s1 phenotype. In contrast to the microinjected thoracic serotonergic neurons, the abdominal s1 does not branch in the lateral neuropil [8]. It is not known if this is an intrinsic or extrinsic property of the development of this cell.

Compared to blocking transcription, microinjecting the translation blocker cyclohexamide gives a similar set of phenotypes. As with blocking transcription, blocking translation before the growth cones have crossed the midline (Figures 3A and 3B) stalls further migration. In addition to one of the serotonergic neurons, a close neuron (probably from the same lineage) was also injected in Figure 3A. Its axon normally extends posteriorly on the ipsilateral side. It also fails to extend an axon. This was also seen for this same neuron when transcription was blocked (my unpublished data). One interpretation of the results of this experiment is that neurons with newly extending axons are more dependant upon nascent gene expression than those whose neurites have extended farther. Once the growth cone has just extended over the midline (Figure 3C), blocking translation results in a phenotype very similar to that resulting from blocking transcription (Figures 3D–3F and 3I). Growth cones in this class continue migration and either extend in an incorrect posterior direction (Figure 3I) or in a number of directions. The extra ipsilateral branch seen in Figures 3D–3F was never seen when transcription was blocked, although in older serotonergic neurons an ipsilateral process is normally present [8]. Another difference between these latter experiments and those of blocking transcription is that serotonin uptake activity is substantially elevated. Optimal fixation conditions for staining serotonin (4% paraformaldehyde) and

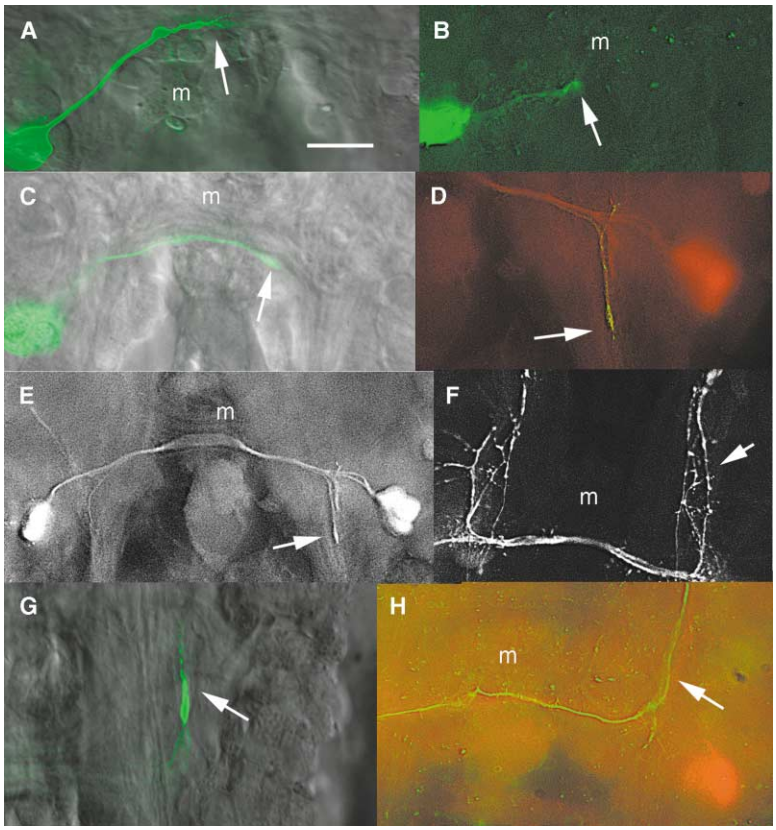

Figure 2. Growth Cone Progression of Transcription Blocker-Injected Serotonergic Neurons

All cells shown in this figure were microinjected with dye and the transcription blocker actinomycin D. (A) A growth cone at the time of microinjection is just on the midline (m). (B) The same growth cone as shown in (A), 24 hr later, has not moved from its original position. (C) At the time of microinjection, the growth cone is between the midline and the contralateral connective. (D) 24 hr later, the same growth cone as shown in (C) has reached the contralateral connective and extended in the wrong direction. (E) A lower-magnification photograph of (D) shows serotonin staining. Note the microinjected growth cones extending in the wrong direction while the control side is extending correctly. (F) Serotonin staining of a microinjected s1 serotonergic neuron (arrow) that had already turned at the contralateral connective at the time of injection has continued to extend in the right direction. However, in contrast to the control side, there is no s1-type bifurcation. (G) A microinjected growth cone has already made the turn in the contralateral connective and 24 hr later (H) has continued to extend anteriorly along the connective. The scale bar in (A) represents 10 μm in (A–C), (E), (F), and (H) and 5 μm in (D) and (G).

dextran dye (2% paraformaldehyde) are not very compatible. Normally, it is difficult to see strong serotonin staining as well as injected dye, but when translation was blocked, activity was greatly elevated (Figures 3F and 3I) such that only the injected side could be seen to be stained for serotonin. Interestingly, isolated serotonergic growth cones show increased serotonin uptake activity, and that activity becomes independent of a critical midline signal [3]. In light of the data shown here, it is likely that there is an unstable protein that blocks serotonin uptake activity early in development. Consistent with this idea, blocking translation after the growth cones have reached the contralateral connective or made the turn does not block further migration (Figure 3H), and as with blocking transcription, no s1-like bifurcations were seen (Figure 3H). A summary of the results of all microinjections conducted is shown in Figure 4.

Both cyclohexamide and actinomycin D are cell permeable, and thus any effects seen might be indirect. However, in the many cases in which only cells nearby s1/2 were injected (Figure 3J), both ipsilateral and contralateral projections are normal. Whatever drugs leak out of cells, it is not enough to affect the axon guidance of the neighboring serotonergic neurons. In a number of cases, when one serotonergic neuron was injected and the other not, as indicated by dye content (see Figure 2D), both had abnormal projections. Either the uninjected serotonergic neuron can pick up drug from a neighboring injected serotonergic neuron or some cooperative interaction occurs between these growth cones at the choice point. It is possible that serotonergic neurons junction more with each other than with neighboring cells, thus increasing specific transfer of the drug.

Blocking either translation or transcription between the midline and the connective choice point gives similar phenotypes. One explanation for this is that a signal from the midline induces the expression of a activity in the neuron critical for correct turning at the connective. The alternative is that intrinsic timing regulates the expression of molecules important for pathfinding. It is estimated that it takes axons about 5 hr to migrate from the midline to the contralateral connective [3, 14], allowing plenty of time for at least one round of gene expression. With the exception of serotonin uptake activity, blocking translation has no greater effect on the serotonergic growth cone migration than does blocking transcription. This indicates that the gene expression events assayed here involve both transcription and translation and argues against a distinct regulatory function for local translation in the growth cone, at least for this one choice point. It is not clear why the misrouted axons grow posteriorly instead of anteriorly. One possible explanation is that growth cones pause on the contralateral connective in wait for another set of anteriorly extending axons. In this scenario, it is in the recognition of these anteriorly projecting pioneer axons that the serotonergics fail. However, cutting any or all of the four connectives does not affect serotonergic axon guidance (my unpublished data). In many of the cases observed and shown in Figures 2 and 3, extra branches are seen. Small posterior extensions are also seen in controls (see Figure 1B). In vivo studies of axons at choice points

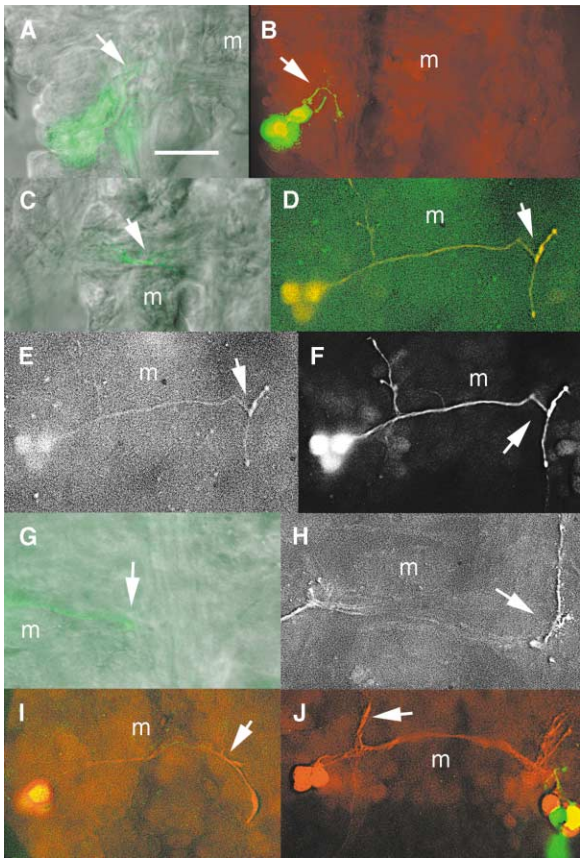

Figure 3. Growth Cone Progression of Translation Blocker/Dye-Microinjected Serotonergic Neurons

(A) A cyclohexamide/translation blocker-microinjected serotonergic growth cone has just started toward the midline. (B) 24 hr later, the same growth cone has not progressed any farther. Another growth cone belonging to a sib of the serotonergic neurons (turning posteriorly and behind the serotonergic growth cone in [A]) has also progressed no farther in (B). (C) A growth cone just over the midline at injection has progressed to the turning point on the contralateral connective in (D). Panel (E) Shows the tracer dye from (D), and (F) shows serotonin uptake. The growth cones have bifurcated on the connective instead of growing anteriorly only. Serotonin uptake in the injected cell is very strong when compared to that in the control side. Occasionally, when serotonin staining is very strong, a third serotonergic neuron appears and has been described before [8]. This third cell behaves very similarly to s2 and is thought to be a sib of s1/2. (G) A growth cone has just arrived at the contralateral turn at injection and after 24 hr (H) has extended toward the anterior segment. (I) A microinjected cell stained for serotonin uptake (red) and tracer dye (green) after 24 hr. This growth cone had just passed the midline at injection time and has made a wrong turn on the connective. In addition, serotonin uptake is significantly stronger than that in control side. (J) Neurons injected with cyclohexamide have not affected serotonin or guidance (arrow) of the nearby serotonergic neurons. (A–I) The scale bar represents 10 μm.

[14–16] have shown that retraction of inappropriate arbors also plays a role in selection of the appropriate pathway. The pathfinding errors seen in this study might therefore represent an inability to stop the growth of inappropriate branches. However, what this data does show is that specific axon guidance events do require the autonomous expression of genes involved in pathfinding.

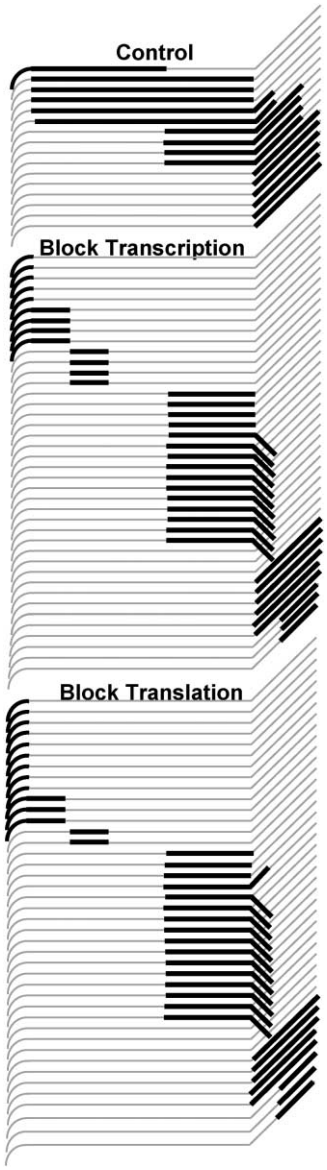

Figure 4. Summary of Growth Cone Trajectories after Microinjection

The light-gray line represents the normal trajectory of the s1/s2 growth cone. The thick black line indicates the path taken after microinjection. The start of the thick black line indicates where the growth cone was at the time of microinjection, and the end of this line is where it gets to after 24 hr. For clarity, only the longest branch for each case is shown. s1/s2 serotonergic growth cones are estimated to take about 10 hr to get to the ipsilateral connective (the first bend in the gray line), another 5 hr to the midline and 5 hr to the contralateral connective, where they pause for about 10 hr (bend on gray line on right-hand side). It then takes about 10 hr for the s1 growth cone to get into the synaptic neuropil where branching begins. The experiments are grouped by control, blocked transcription, or blocked translation. The main differences between controls and those blocking gene expression are failure to cross the midline and failure to make the connective turn correctly. Growth cones in only one experiment in which translation was blocked made the correct turn.

Experimental Procedures

Grasshopper embryos were obtained from a crowded colony maintained in the lab as described [3]. Embryonic CNS's were staged, dissected, and cultured as described [3]. Microinjection was performed as described [3]. Actinomycin A (100 μg/ml, Sigma) or cyclohexamide (20 μg/ml Sigma) were injected into neurons along with FITC-dextran tracer dye (D-1817, Molecular Probes, 25 μg/ml). Injections were made only into the left thoracic serotonergic neurons. Imaging was performed on an Olympus BX40 microscope, and photographs, DIC and fluorescent, were taken with a Photometrics SenSys camera with an Olympus 40× or 20× lens. ImageIP software was used for capturing the images. Fluorescent images were subjected to one frame deconvolution (95% removal, 75% gain) with VayTek Hazebuster. Fluorescent channels were stacked in Adobe Photoshop, and layouts/labeling were performed in PowerPoint. Anti-serotonin was from Immunostar (#20080) and was used at 1/1500; secondary antibodies were purchased from Jackson labs and were used at 1/300. Staining procedures were as described [3].

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